JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2005, p. 4197–4199 Vol. 43, No. 8
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.8.4197–4199.2005

Approaches to Bacterial RNA Isolation and Purification for Microarray Analysis of *Escherichia coli* K1 Interaction with Human Brain Microvascular Endothelial Cells

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Received 5 March 2005/Accepted 10 May 2005

We established a protocol for isolation of microarray-grade bacterial RNA from *Escherichia coli* K1 interacting with human brain microvascular endothelial cells. The extracted RNA was free of human RNA contamination. More importantly, microarray analysis demonstrated that no bias was introduced in the gene expression pattern during the RNA isolation procedure.

DNA microarrays offer new opportunities for exploring the molecular pathogenesis of infectious diseases. It is possible to analyze the whole gene expression of a bacterial pathogen during its interaction with the host. Such information can lead to the identification of virulence factors, the elements to which they respond, and the mechanisms by which they are regulated (1, 2, 6, 14, 15, 17). The feasibility of this approach, however, depends upon the ability to recover biologically relevant bacterial RNA, and serious consideration should be given to prevent gene expression changes associated with preparative procedures (6, 10). This is a difficult task, since bacteria are capable of rapid transcriptional responses to the environment, a fact extensively mentioned in the past but hardly addressed in full. Moreover, RNA must be obtained from biologically relevant models of infection that reflect the complexity of pathogen-host interaction. Such models are amalgams of pathogens and host cells, which make DNA microarray analysis even more challenging.

We developed a protocol for RNA extraction from *Escherichia coli* K1 interacting with human brain microvascular endothelial cells (HBMEC). HBMEC represent the in vitro model of the human blood-brain barrier, and we have previously demonstrated that *E. coli* K1 interaction with HBMEC is a biologically relevant model pertaining to the pathogenesis of *E. coli* meningitis in vivo (7–9, 19, 20). We initially attempted to coextract bacterial and human RNA from a mixed sample of *E. coli* K1 and HBMEC. This approach did not require time-consuming steps that can affect bacterial gene expression but creates other technical issues for microarray analysis, as human RNA can compete with bacterial RNA during cDNA synthesis and labeling, limit the amount of bacterial RNA that can be used, and impede accurate quantitation of bacterial RNA. Moreover, the ratio between host and pathogen RNA may vary among samples, making normalization of microarray data difficult. Thus, it was necessary to eliminate the human RNA, which could be done by hybridization capture using MICROBEnrich (Ambion). Total RNA was extracted from HBMEC and associated bacteria a using RiboPure-Bacteria kit (Ambion) that includes zirconia-silica beads for bead beating with a special vortex adapter (Ambion). Bead beating was necessary to lyse *E. coli* K1. Human RNA extracted using this method was found to be degraded, whether extracted from the mixed HBMEC-bacteria sample (28S/18S = 0.33; Fig. 1A) or a pure HBMEC culture (28S/18S = 1.2; data not shown). RNA extracted from a pure culture of *E. coli* K1 (data not shown) produced high quality RNA as expected (23S/16S = 1.8). Degraded human RNA cannot be removed by hybridization capture and could affect microarray analysis.

As the coextraction protocol did not produce microarray-grade RNA, we developed an alternative method using differential lysis. HBMEC were eliminated using RLT lysis buffer (QIAGEN) that caused immediate and complete lysis of HBMEC but did not affect *E. coli* K1 (Fig. 2). Intact bacteria were separated from HBMEC quickly to avoid any alteration of

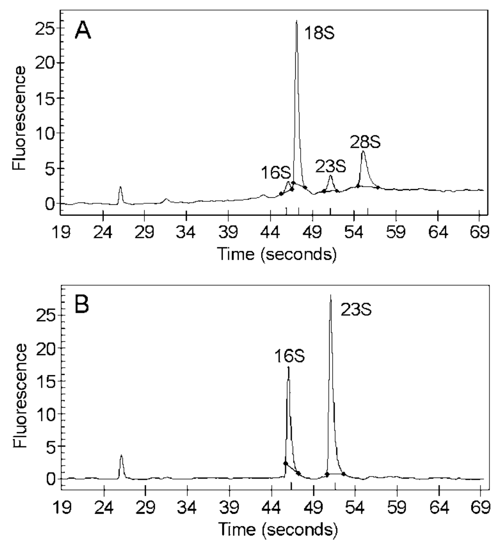

FIG. 1. Agilent 2100 bioanalyzer electropherograms of RNA samples extracted from HBMEC and *E. coli* K1 strain RS218 using the coextraction protocol (A) or the differential lysis protocol (B).

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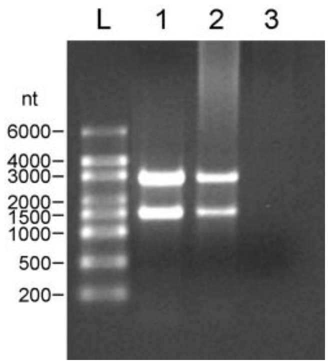

FIG. 2. Native agarose gel electrophoresis of total RNA extraction from *E. coli* K1 strain RS218 using different lysis buffer and treatments. Lane L, RNA ladder, high range (Fermentas, Hanover, MD); lane 1, RiboPure-Bacteria lysis buffer with supplied zirconia beads (Ambion); lane 2, RLT lysis buffer plus glass beads (RNeasy Mini kit; QIAGEN; acid-washed glass beads, 150 to 210 μm; Sigma); lane 3, RLT lysis buffer, no beads (RNeasy Mini kit; QIAGEN). *E. coli* K1 RNA could be extracted only when bead beating was applied (lane 1 and 2, bands of rRNAs are clearly visible) and not when RLT lysis buffer (QIAGEN) was used without bead beating (lane 3, no rRNA bands are visible). nt, nucleotide.

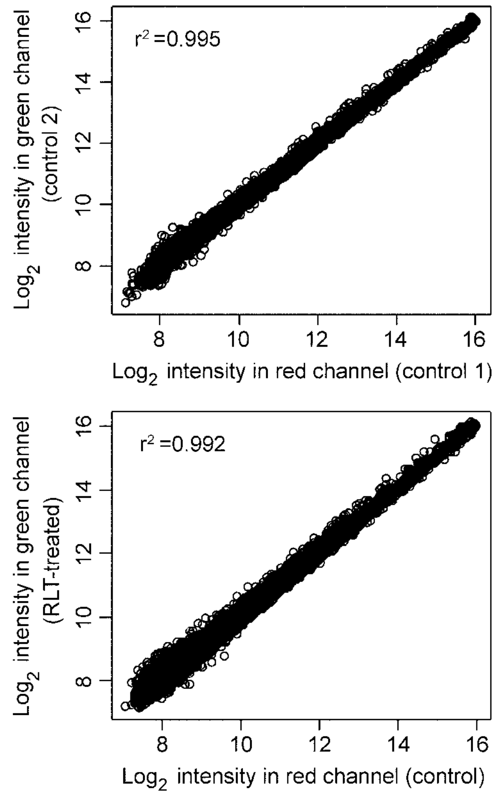

FIG. 3. Scatter plot of fluorescence intensity and correlation coefficients from DNA microarray analysis of untreated and RLT-treated *E. coli* RS218. Functions of a *limma* package from Bioconductor (http://www.bioconductor.org, an open-source project implemented in statistical language R) were used. Top panel, self-self hybridization of untreated *E. coli* RS218 (reference sample). Bottom panel, hybridization between untreated (reference) and RLT-treated *E. coli* RS218 (5 min).

bacterial gene expression. This was necessary to obtain a sample that was representative of *E. coli* K1 interacting with HBMEC. HBMEC infected with *E. coli* K1 strain RS218 (18) were prepared as previously described (13) in 150-mm dishes. Nonassociated bacteria were collected from the culture supernatant by centrifugation and resuspended in RLT buffer (QIAGEN, Valencia, CA). The infected monolayers were then washed once with M199-Ham F12 (1:1; Invitrogen), resuspended in RLT buffer (QIAGEN), and immediately collected from the plates. Both suspensions were vortexed for 1 min and centrifuged for 1 min at high speed. The supernatant (RLT buffer with or without HBMEC lysate) was discarded, and the bacterial pellet was extracted using a RiboPure-Bacteria kit (Ambion). RNA was cleaned up and concentrated using an RNeasy Mini kit (QIAGEN) with on-column DNase treatment. Samples that were processed with this method yielded ~25 μg of bacterial RNA of outstanding quality, free of human RNA contamination (Fig. 1B). This bacterial RNA sample was found to be suitable for microarray analysis. We compared the expression profiles of associated and nonassociated bacteria using a DNA microarray designed in our laboratory. Our *E. coli* DNA microarray consists of 8,239 oligonucleotides (50-mer) arrayed onto aminosilane slides (UltraGAPS; Corning) and covers each open reading frame in *E. coli* K-12 strain MG1655, *E. coli* O157:H7 strains EDL 933 and RIMD0509952/VT2-Sakai, most open reading frames in uropathogenic *E. coli* strains (CFT073, 536), and meningitis-causing *E. coli* strains (RS218, C5). This DNA microarray analysis was carried out as previously described (21).

Differential lysis was previously applied to pathogen-host systems (3–5, 10–12, 16). However, it was unclear whether or not bacterial gene expression could be altered during differential lysis. Applying RNA stabilization reagents (RNA*later*, Ambion; or RNAprotect bacteria; QIAGEN) caused a carryover of degraded host RNA (data not shown) which was not suitable for microarray analysis. We examined, using our *E. coli* DNA microarray, whether our differential lysis protocol could introduce any bias in the bacterial gene expression pattern. *E. coli* RS218 in early-log phase was prepared and split into three aliquots. One was immediately extracted (reference) while the others were treated with RLT buffer (QIAGEN) for 5 or 15 min to simulate differential lysis. Except for the absence of HBMEC, RNA was extracted from these samples as in our differential lysis protocol. Samples were compared using our *E. coli* microarray as previously described (21). The expression pattern of bacteria incubated in RLT buffer for 5 min was nearly identical to that of untreated *E. coli* RS218 (Fig. 3), and the correlation coefficient (r^2) was essentially indistinguishable between a reference-reference self hybridization (0.995) versus a 5-min-RLT-treated–reference hybridization (0.992). In contrast, longer RLT buffer treatment (15 min) resulted in higher variability, and more than 90 genes appeared differentially expressed (data not shown). Based on these findings we concluded that *E. coli* K1 gene expression was not significantly altered during a 5-min differential lysis treatment with RLT buffer. Our protocol easily allows the procedure to stay within this time limit. Indeed, a few seconds are sufficient to completely lyse the HBMEC. Most mammalian cell lines will be lysed equally fast, thereby our protocol can be applied to a variety of bacteria-host infection models as several bacteria can withstand a short incubation in RLT buffer. Other lysis buffers can also be applied providing that preservation of bacterial gene expression is properly verified.

This work was supported by National Institutes of Health grants NS-26310 and AI-47225.

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